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## The Biophysics of Nucleic Acids Sensing by Hybridization on a Lab-on-Chip Device

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**Abstract:** An analysis of the oligonucleotides hybridization efficiency and mechanisms, by means of fluorescence detection, on the Lab-on-Chip device provided by STMicroelectronics, is here reported. The device consists of a silicon oxide microfabricated reactor capable to perform a PCR, and fluidically integrated with a microarray detection module aimed to detect the specific amplified DNA, *via* hybridization, with an assay-specific DNA microarray printed on the silicon oxide surface. The study was performed by using two different silicon surfaces featured by diverse amount of silanol (Si-OH) groups. The study put in evidence the relationship between such type of surface differences with DNA oligonucleotide surface probe density and the device analytical sensitivity. The sensitivity curves and the thermodynamics of a model system have been carried out. *Copyright* © 2012 IFSA.

**Keywords:** DNA Microarray, Hybridization, Microfabricated silicon chip, Silanols, Lab-on-Chip, PCR.

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### 1. Introduction

In the interest of developing rapid and portable diagnostic tests, the microfabricated chip is an attractive format for miniaturizing multiple preparatory and analytical steps in what is called a “laboratory on a chip” (lab-on-a-chip) device. One major area of development of lab-on-a-chip devices is in the field of genotyping and molecular diagnostics, in which all the stages from preparation of DNA, passing through amplification to the assignment of species, are integrated into a single device. Ideally, such type of lab-on-a-chip device requires only a limited amount of biological material and small volumes, and provides results in a relatively short time. Over the last decade a number of prototypes microfabricated devices have been developed to perform one or more steps of nucleic acids

analysis, in particular using polymerase chain reaction (PCR). The reaction chambers of these “biochips” are often produced from silicon wafer [1], but some are composed of glass [2], plastic [3] or polycarbonate. Some of these innovative devices associate PCR with post-amplification analysis such as electrophoresis [2] or microarray detection [4].

DNA microarrays are analytical devices designed to determine the composition of multicomponent solutions of nucleic acids [5]. STMicronics has developed a new PCR and microarray integrated silicon biochip, as consumable of the molecular diagnostics platform *In-Check*, provided by STMicronics as well. The particular architecture of the device guarantees to miniaturize reaction volumes while simultaneously reducing amplification times. The microarray is used to hybridize and detect the DNA coming from an amplification step via PCR, occurring in the embedded silicon heated chambers. The detection is based on standard fluorescent labeling of PCR product by using Cy5-dCTP incorporation or Cy5-labeled primers. The amino modified probes constituting the microarray are printed on the epoxysilane coated amorphous silicon oxide surface, through amino bonds. The protocol of analysis comprises also the use of some Cy5-labeled deoxioligonucleotides (DNA oligonucleotides) perfect match, used as hybridization controls to check if the hybridization reaction properly occurs. Of course, physical-chemistry of hybridization processes is crucial for the effective developing of protocols for diagnostics purpose.

In this paper we focused on the thermodynamic aspects of the hybridization processes occurring between deoxioligonucleotides perfect-matches in solution and the probes grafted onto the surface, discussing the hybridization isotherms by means of the Langmuir model. Moreover, different silicon surfaces with various amount of silanols (Si-OH) terminations, whose surface density is critical for a proper probes grafting, have been compared with the aim to provide more information about the effect of surface features on the microarray detection performances (i.e. sensitivity). The hybridization process is influenced by several factors at the solid-liquid interface such as surface heterogeneity, surface charge, non specific adsorption and probes density. These parameters can strongly affect the hybridization efficiency [6]. In fact, in general, surface equilibrium constants appear to be depressed if compared with solution values and the above listed parameters result critical factors for the efficiency of the hybridization affecting, together with the efficiency of PCR, the overall diagnostics ability.

## **2. Materials and Methods**

### **2.1. Materials**

#### **2.1.1. Chemicals**

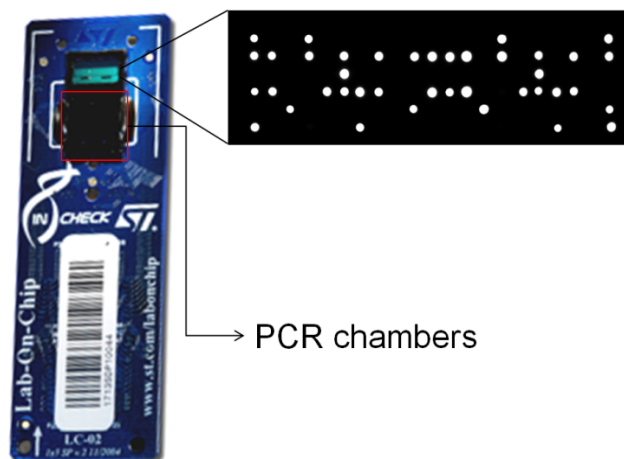
The following reagents were used for the study. Hydrogen peroxide (29 %) ammonium hydroxide (25 %) hydrochloridric acid (37 %) and methanol for wet processes were purchased by Sigma Aldrich and were used as received. Anhydrous toluene and glycidoxy-propyl-trimethoxy-silane (GOPS) for silanization step were purchased by Sigma Aldrich and were used as received.

#### **2.1.2. Chemicals for Passivation Steps**

Sodium Dodecyl Sulfate and Sodium Citrate from Sigma Aldrich and were used as received. Bovine serum albumin (BSA) fraction V has been purchased from Euroclone and was used as received. All DNA oligonucleotides were purchased from MWG Biotech: the probes for grafting onto the Lab-on-Chip surface were desalted while Cy5-labeled target (perfect match) were HPLC purified.

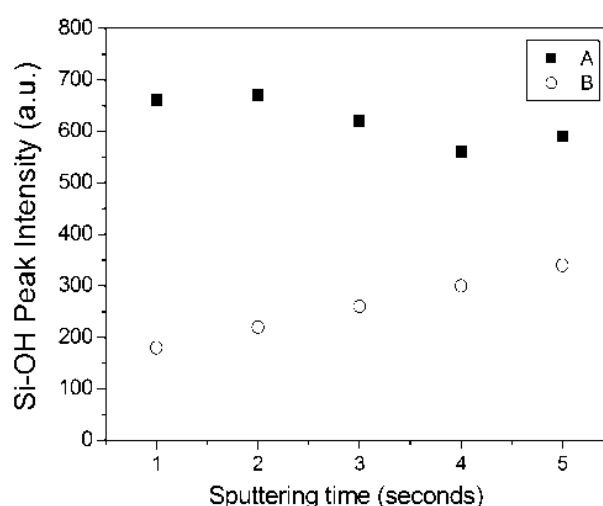
### 2.1.3. Substrate

The STMicroelectronics Lab-on-Chip (LoC) is a silicon-based MEMS (Micro Electro Mechanical System) device (Fig. 1). It was used as substrate of testing. It consists of a silicon chip mounted on a PCB board featured with electrical pads to be actuated with external instruments. The device is characterized by two embedded silicon chambers aimed to perform the PCR and by an exposed detection area featured by a 25 mer nucleic acids probes microarray.



**Fig. 1.** STMicroelectronics LoC. A typical microarray image has been reported as example of hybridization outcome.

Two different substrates featured by diverse superficial silanol (Si-OH) content have been used for hybridization experiments. For sake of simplicity they have been named A and B, in which A is the richest in Si-OH terminations, as showed in ToF-Sims reported (Fig. 2). The B differs from A because it underwent to a surface cleaning process through O<sub>2</sub> plasma before the wet chemically processing to functionalize the surface with epoxysilane.



**Fig. 2.** TOF-Sims analysis on silicon group A *versus* group B. A is the group without plasma cleaning. B with plasma cleaning.

#### **2.1.4. Equipments**

Teflon tanks for wet processes were manufactured by SPM. Glove box in nitrogen atmosphere used for silanization process was purchased from BRAUM. Customized piezo spotters have been used in class 100 for microarray printing. Temperature Control System, named TCS (it is provided by STMicroelectronics as part of the In-Check platform), has been used for hybridization experiments. A CCD camera-based Optical Reader (it is provided by STMicroelectronics as part of the In-Check platform) has been used for in-fluorescence images acquisition.

### **2.2. Method**

#### **2.2.1. Surface Derivatization**

- Substrate cleaning and activation processes:  
silicon oxide surface was cleaned by cleaning process ( $\text{H}_2\text{O}:\text{NH}_4\text{OH}:\text{H}_2\text{O}_2$ ) in a Teflon tank, rinsed by deionized water and dried by nitrogen flow; the activation step was carried out using an acid methanol solution  $\text{CH}_3\text{OH}:\text{HCl}$ , rinsed by deionized water and dried by nitrogen flow.
- Substrate silanization processes:  
The activated silicon oxide surface was dried by nitrogen and silanized in nitrogen atmosphere by immersing the substrates in a GOPS anhydrous solution. Silanized substrates were stored in vacuum condition until the amino modified deossinucleotides printing.
- Deossiologonucleotides printing on surface:  
High throughput customized piezo spotter has been used to deposit micro drops of about 200 pL, containing amino-modified micro molar oligonucleotides
- BSA passivation coating:  
Printed substrates were immersed in BSA aqueous solution with the aim to passivate the surface and to remove the excess of not grafted amino-modified deossinucleotides on the surface. Substrates were then rinsed in deionized water and dried by a nitrogen flow.  
All processes above described were performed in a clean room (class 100)

#### **2.2.2. Microarray Content**

The microarray layout of probe arrangement on the detection area was specifically designed. It consists in a panel of DNA oligonucleotides probes arrayed in a matrix of  $6 \times 21$  spots. The layout contains probes of AT series (*Arabidopsis Thaliana*)

#### **2.2.3. Hybridization experiments**

For the sensitivity experiments a total of 24 LoCs were used, split into 12 for each A and B. The 24 LoCs were chemically treated in a homogeneous batch according to the recipe described in surface derivatization section.

The sensitivity of the microarray was evaluated by hybridization with Cy5-labeled perfect-matches (simply named as target). Six different concentrations of target in solution (0.005-0.05-0.5-15-30-100 nM) have been used to generate the sensitivity curves on both substrates. The fluorescence acquisition (excitation at 630 nm and emission acquisition at 670 nm) has been performed by using the

CCD camera-based Optical Reader (part of the *In-Check* platform). Images were acquired by using different shutter times to avoid signal saturation when testing high concentration of target. At the end, results were normalized and expressed as number of fluorophores per surface unit. The number of fluorophores per surface unit has been calculated by using a calibration curve of target concentration versus the signal intensity, by correlating the signal intensity of target drops dispensed onto the LoC surface. To build such calibration curve, in a separated experiment solutions at various Cy5-labeled oligonucleotides were dropped onto the silicon surface of Lab-on-Chip and signal fluorescence was recorded at various shutter times.

The hybridization protocol consisted of a loading step of the hybridization mix put into the detection area surface, containing the microarray panel with a successive sealing of the area with suitable clamps, appositely manufactured and provided with the *In-Check* platform. The hybridization mix is composed by the Cy5-labelled oligo perfect match at various concentrations.

### 3. Theoretical Considerations and Experimental Constrains

At very low DNA concentration, hybridization sensitivity curves can be generally described by the simplified equation

$$F_x = K_b [T \text{ arg et}] \quad (1)$$

where  $F_x$  is the number of fluorophores hybridized, [target] is the concentration of DNA target in solution and  $K_b$  the proportional constant.  $K_b$  is just the slope of equation (1) when [target]  $\rightarrow 0$ . It depends on both  $K_{eq}$  and surface features such as DNA binding density, surface charge, etc.

Thus,  $K_b$  allows the assessment of the specific probe sensitivity. Obviously, the higher is the  $K_b$  value the higher is, at the same target concentration ([target]), the  $F_x$  for probe.

The hybridization curves should follow the Langmuir isotherm that is described by the equation (2), although some requirements must be satisfied such as the measurements performed in a state of equilibrium and the need of a moderate spot density to avoid steric hindrance.

Moreover, additional conditions [7] must be accomplished to fit the Langmuir model, such as:

- Each spot must contain only one type of probe;
- Each probe must be perfectly selective;
- A target cannot hybridize with complementary strands in solution;
- Each target hybridizes with a single probe.

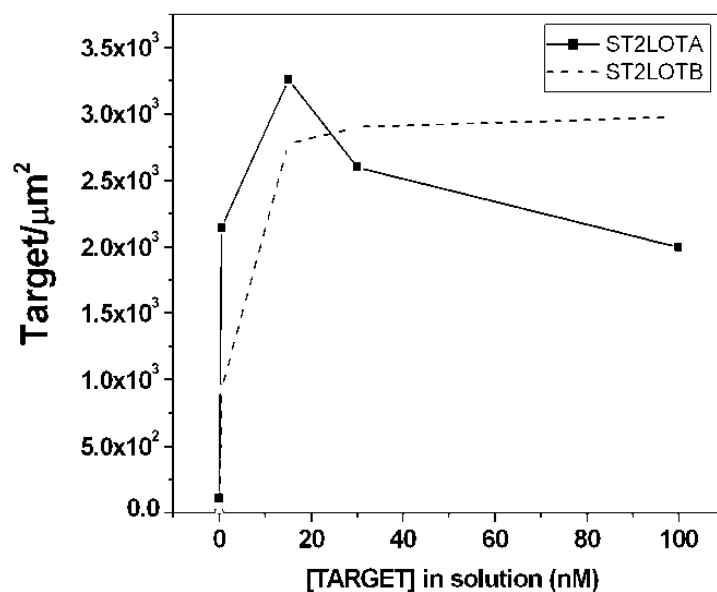
The Langmuir model predicts the presence of a linear regression region at low concentration of DNA target and a plateau region over high target concentration. At very low DNA concentration, the Langmuir equation is [8]:

$$\frac{F_x}{F_{100} - F_x} = K_{eq} [T \text{ arg et}] \quad (2)$$

where  $F_x$  is the number of hybridized sites (DNA probe) at a specific target concentration in solution;  $F_{100}$  is the values of fluorophores in the plateau region of the plot, at the saturation of the hybridization sites;  $K_{eq}$  is the hybridization equilibrium constant (that is the ratio between the kinetic constant of hybridization ( $k_{\text{hybridization}}$ ) and kinetic constant of de-hybridization ( $k_{\text{de-hybridization}}$ )).

## 4. Results and Discussions

The microarray sensitivity has been assessed through hybridization reactions with different concentration of perfect-match Cy5-labeled oligonucleotides. After the hybridization experiments, the obtained signal intensity data were converted in target concentration values of fluorophores per surface unit ( $F/\mu\text{m}^2$ ) through calibration curves for the specific time acquisition setting. The obtained density values were plotted versus the related values of concentrations of target in solution. Fig. 3 shows the hybridization isotherm curves for one of the AT probes (internally named ST2) reporting the concentration of target in the spot after hybridization ( $\text{Target}/\mu\text{m}^2$  in axis Y) versus the concentration of target in the hybridization reaction ( $[\text{TARGET}]$  in solution (nM) in axis X).



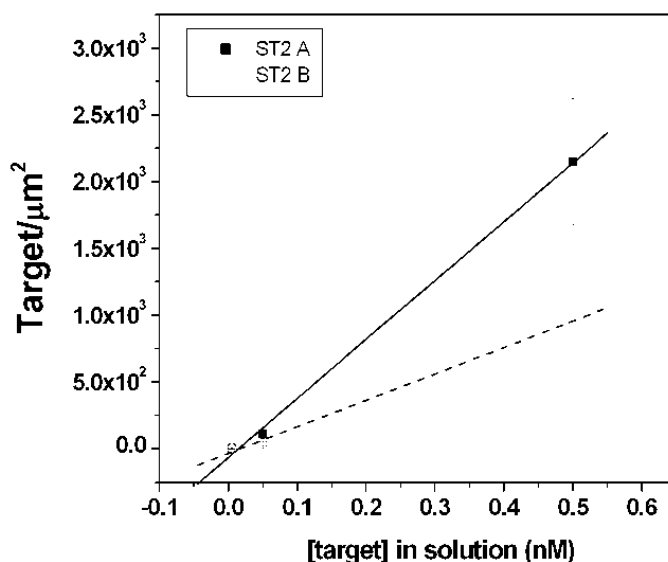
**Fig. 3.** Equilibrium isotherms for hybridization of ST2 probe of both samples groups A and B.

According to the above isotherms, both the plots are characterized by a linear region (from 0 to about 0.5 nM), showed in Fig. 4, reaching a maximum around 15 nM.

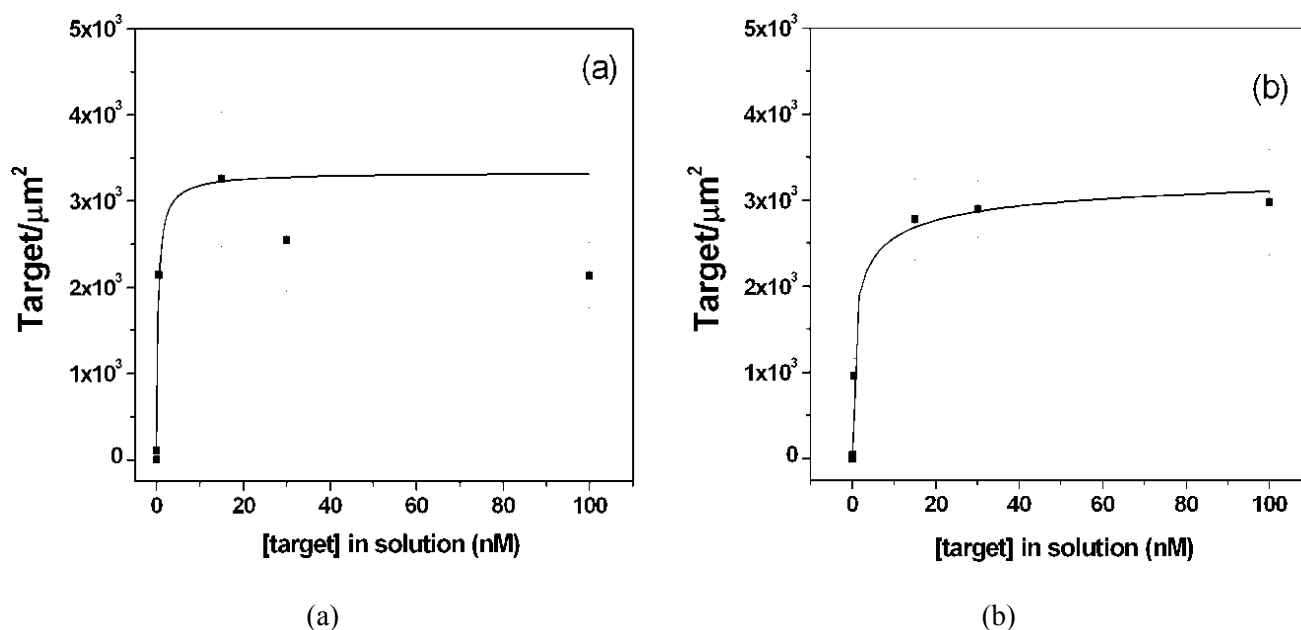
The plateau region indicates that all the probe sites available and accessible for hybridization are saturated by the DNA. At this regard, it should be noted that, for samples of group A, in contrast to what predicted by a Langmuir model, the plateau zone does not coincide with the observed maximum value of the target concentration in the spot but decreases when the target concentration is enhanced. The discrepancy is well observed by comparing the Langmuir fit for each probe plot in Fig. 5 and the value at saturation.

Differently, for samples of group B the plateau zone basically coincides with the observed maximum value of the target concentration in the spot, as predicted by the Langmuir model.

As reported in Materials and Methods section, as regards the substrate characterization, the ToF-Sims analysis reveals that the substrate belonging to group A has a content of Si-OH group three times and half more than samples from group B, at 1 sec of argon sputtering. This is reasonable since plasma treatment can reduce the amount of silanols on the surface [9]. Thus, since Si-OH content directly influences the density per area unit of the epoxy-silane molecule to which oligo probes are covalently bonded, this clearly indicates that the probe density is higher in the samples from group A than samples from group B.



**Fig. 4.** Linear regions (low target concentration) of equilibrium isotherms for hybridization of ST2 probe of both samples groups A and B.



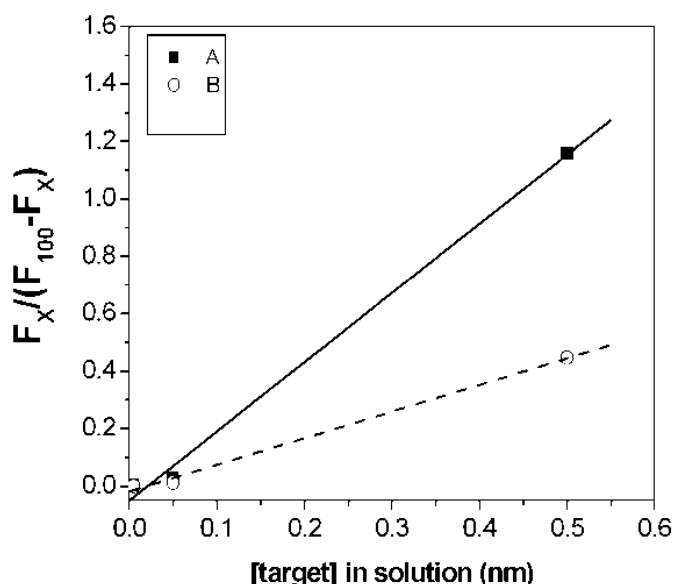
**Fig. 5.** Individual hybridization isotherms (scatter) and Langmuir fit (solid line) for ST2: (a) Samples from group A; (b) samples from group B (b).

As consequence, the steric hindrance is not negligible and can reduce the target molecules accessibility. This last can be influenced not only by the simple steric effect but also by electrostatic repulsion, generated by the probe to probe proximity, whose entity directly depends on the inter probes distance [10, 11]. As reported in literature [12, 13], the hybridization efficiency generally decreases while the probe density increases. In literature is reported that the right compromise between high probe density, that from one side should guarantee higher sensitivity but with risk for target accessibility, and low density, yielding lower sensitivity but coherent analyte/signal response, should be studied and opportunely set [14]. Indeed, in our case, probe density influences the kinetics and thermodynamics of the reaction yielding results that cannot be fit with Langmuir model. Additionally, It is also possible to suppose that, together with steric hindrance and electrostatic effect, some static self-quenching phenomena can occur between the Cy5 units of the target molecules hybridized to the

probes in the spot that, being closer than in the group B, at the highest concentrations of the target determine emission quenching, with consequent lower emission intensity than group B samples. This leads to underestimate the amount of target that hybridizes the related probe with the increasing of target concentration in solution.

In order to determine the amount of fluorophores in the plateau region of the plot, at the saturation of the hybridization ( $F_{100}$ ), to carry out the  $K_{eq}$  for both the samples groups, the fitting of the experimental data has been performed as reported in Fig. 5. Although the samples from batch A drift from the theoretical model, it can be assumed that results at low concentration of target in solution up to 15 nM are quite coherent, as reported in Fig. 5, where the linear region is in accordance with the expected behavior. Indeed, due to the fact that at low target concentration the steric hindrance is negligible,  $K_b$  for batch A is higher than batch B (Fig. 4) and this is reasonable since the probe density is higher t.

Fig. 6 reports the plot of the ratio  $F_x/(F_{100}-F_x)$  versus the concentration of target in solution. As described in section 3.1, the slope of this linear plot is the hybridization equilibrium constant  $K_{eq}$  when the  $[target] \rightarrow 0$ . In Table 1 the values of equilibrium constants, calculated by plot in Fig. 6, and the related sensitivity constants, calculated by plot in Fig. 4, have been summarized.



**Fig. 6.** Plot of  $F_x/(F_{100}-F_x)$  versus the  $[target]$  (nM). The slope is the  $K_{eq}$ .

**Table 1.** Summary of the equilibrium ( $K_{eq}$ ) and sensitivity ( $K_b$ ) constants for probe ST2 determined on both group A and B.

ST2 PROBE	$K_{eq}$ $10^9 * L * mol^{-1}$	$K_b$ $(10^{-8} * mol * L^{-1}) / cm^2$
Group A	2.4	7.5
Group B	0.9	3.3

## 5. Conclusions

It has been investigated the effect of the surface properties on the hybridization reactions efficiency, studied on the STMicroelectronics Lab-on-Chip device. In particular, the main focus was on the investigation of the role of surface silanols amount in the achieving of a proper and effective surface

silanization process, fundamental for the eventual grafting of DNA amino-modified oligonucleotides. A well tuned grafted DNA surface density has been revealed to be crucial for the sensitivity of the platform and the coherence with the Langmuir model. Indeed, a too high concentration per surface area of DNA amino-modified oligonucleotides, although being in principle useful to guarantee higher sensitivity, was found to be responsible for a not appropriate sensitivity response due to a deviation from the classical Langmuir behavior. For all the above reasons, the tuning of the proper amount of silanols must be carefully addressed to obtain hybridization in accordance to the theoretical answer to optimize the analytical performances of such type of diagnostic device.

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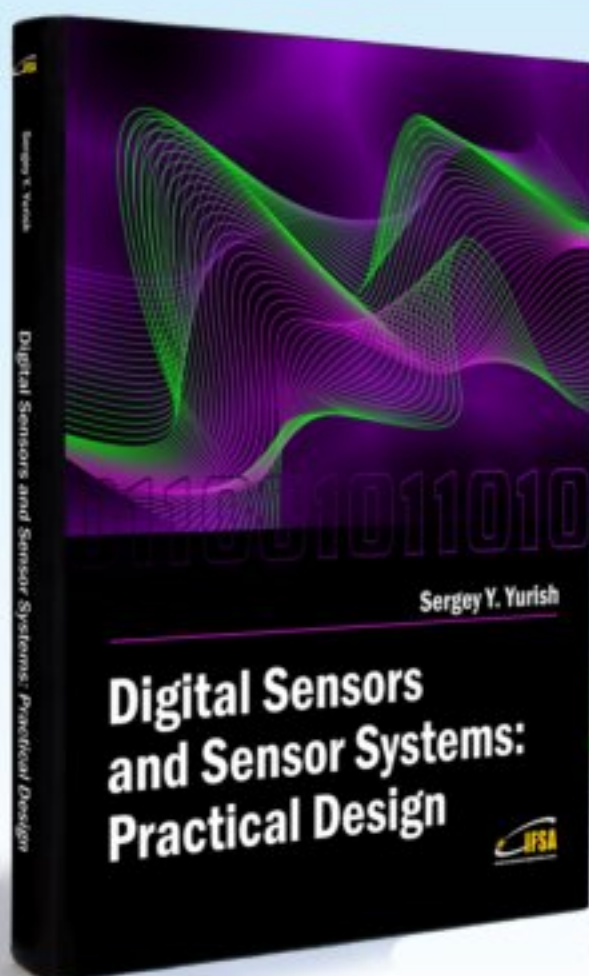
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